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Low-Penetrance Branches in Matrilineal Pedigrees with Leber Hereditary Optic Neuropathy

To the Editor:

Leber hereditary optic neuropathy (LHON; MIM 535000) is an inherited form of bilateral optic atrophy in which the primary etiologic event is a mutation in the mitochondrial genome (reviewed by Riordan-Eva et al. 1995; Nikoskelainen et al. 1996; Howell 1997*a*, 1997*b*). It has been recognized, since the earliest studies of LHON (Leber 1871), that the penetrance is incomplete. It is now understood that this incomplete penetrance reflects a complex etiology and that multiple secondary factors modify or determine the manifestation of the optic neuropathy in LHON (reviewed by Howell 1997*a*, 1997*b*).

The identification of these secondary etiologic factors has been difficult, but heavy smoking and alcohol consumption have received epidemiological support (e.g., see Johns 1994). It appears, however, that there are numerous, but poorly defined, physiological, environmental, societal, and demographic "life style" factors that modify the risk of optic neuropathy. For example, there has been a relatively recent (i.e., during the second half of this century) parallel decline in penetrance among Australian LHON families and in the incidence of a pathologically similar, *acquired* optic-nerve disorder, tobacco-nutritional amblyopia. This trend suggests that there is a common factor in their etiology (Mackey and Howell 1994). In addition, penetrance in LHON families from different northern European countries varies more than twofold (e.g., see Mackey et al. 1996). Even within a single country, such as Australia, there are substantial penetrance differences among 11778 LHON families (Howell et al. 1993).

We have been analyzing penetrance in large, multigeneration Australian and British LHON families, as one approach to the elucidation of these secondary etiologic factors. A previously undescribed pattern of results was obtained during this survey, and we describe here the occurrence of distinct low- (and high-) penetrance branches in LHON pedigrees.

The TAS1 LHON family is the largest matrilineal pedigree that has been assembled. It spanned 11 generations by the early 1990s, and it now comprises >1,600 maternally related individuals, all of whom are descended from a woman who was born in 1777 (III-12 in fig. 1; also see Mackey and Buttery 1992). This LHON family carries the primary mutation at nucleotide 11778 of the mitochondrial ND4 gene (Mackey 1994). Because this family has been located within a relatively small geographical area, because of the good clinical record keeping, and because of the high level of compliance and cooperation on the part of the family, we are confident about the identification of affected and unaffected family members. However, there is an inherent uncertainty in all studies of LHON penetrance, which results from the variable and unpredictable age at onset, spanning the 1st through 8th decades, with a mean in the mid 20s (e.g., see Riordan-Eva et al. 1995; Nikoskelainen et al. 1996). Therefore, LHON carriers (especially males) are always at risk, and there is no age at which one can state with absolute confidence that a family member will remain unaffected.

To control, as much as possible, for the confounding factors in the analysis of penetrance, we have applied the following guidelines. In the first place, we limited our penetrance calculations to *males* who were >30 years of age, to include only those individuals who were past the age of maximum risk. The number of affected females is generally too low, even in the largest LHON families, to provide robust information on penetrance, and they were excluded from the present study. Second, we define here "affected" and "unaffected" in terms of a significant vision loss whose characteristics are compatible with LHON. There are subtle, subclinical

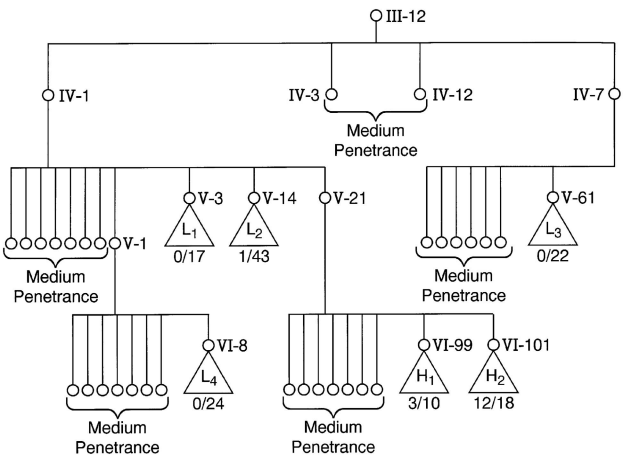

Figure 1 High- and low-penetrance branches in the TAS1 11778-mutation LHON family. This partial pedigree has been drawn to show the genealogical origin of the branches in which there is an unusually low (L1–L4) or high (H1 and H2) penetrance of the optic neuropathy in male family members. Some of the female origins of family branches are shown, with pedigree designations that follow standard numbering schemes (in which the generation designation is denoted by a Roman numeral). The fractions beneath the low- and high-penetrance branches refer to the number of affected (numerator) and total (denominator) males within that particular branch.

changes in the eye (most prevalently, a microangiopathy; see the discussion in Riordan-Eva et al. 1995; Nikoskelainen et al. 1996) that are found at high frequency in LHON family members, but these are not considered here. Ongoing clinical studies of the TAS1 LHON family give no indication that the present results are biased by a high frequency of atypical or unreported ophthalmological abnormalities. Finally, significant recovery of vision is very rare in 11778-mutation LHON patients (reviewed in Howell 1997*a,* 1997*b*), and there is no indication that the penetrance frequencies in the TAS1 LHON family have been biased by this phenomenon.

Analysis of the TAS1 pedigree revealed that there are low-penetrance four branches (designated "L1"–"L4"), in which the penetrance of the optic neuropathy has essentially dropped to zero (fig. 1). A branch is defined here as the descendants of any female in a matrilineal pedigree; the descendants span at least four generations, to provide sufficient information for the determination of penetrance. There is only a single affected male among the L1, L2, L3, and L4 branches, which include a total of 17, 43, 22, and 24 males, respectively. This individual lost vision soon after suffering head trauma in an automobile accident, a severe precipitating factor. For comparison, we ascertained the penetrance in the more typical (designated here as "medium-penetrance") branches of the pedigree. Whereas the L2 branch (which starts with female VI-18) contained 1 affected male among a total of 43, there were 9 affected males, among a total of 53, in the branch that descended from female V-7 and that spans generations VI–IX (this female is not designated in fig. 1). This difference in penetrance frequencies is statistically significant ($P < .05$; 2×2 χ^2 test, adjusted for continuity). These statistical tests must be treated with caution, however, because it is difficult to rule out post hoc bias in the identification of low-penetrance branches. We attempted to address this concern by further analysis of penetrance in the TAS1 pedigree. Thus, the L4 branch is one of several branches that descend from female V-1, and the penetrance is ~12% among males in the other branches that descend from her. In a similar fashion, the penetrance among the descendants of females V-18 and V-57 is 15% and 16%, respectively (these females are not designated in fig. 1). Female V-21 gave rise to two branches if one distinguishes the descendants from her two marriages, and the approximate penetrance values are 33% (which includes the H1 and H2 branches; see fig. 1 and the results given below) and 20%. Therefore, in the comparison of branches of similar size, the low-penetrance branches stand out clearly, a result that argues against severe bias.

The evidence for low-penetrance branching is further supported when the results for all four branches are pooled and the results are compared with the overall penetrance in the matrilineal pedigree. Thus, there is 1 affected male among the total of 106 males in the four branches (a penetrance of ~1%), whereas there are ~200 affected males among a total of ~800 in the medium- and high-penetrance branches of the TAS1 pedigree (an overall penetrance of ~25%). The actual difference in penetrance values is larger, because the estimate of 25% is not adjusted upward to account for those males who are <30 years of age.

There may also be high-penetrance branches, although, because of the small number of family members in these branches, this possibility is less robust. There are in the TAS1 family two small branches (designated "H1" and "H2") in which the penetrance was unusually high. Thus, in the small H2 branch, 12 (67%) of 18 males were affected. Only 3 (30%) of 10 males were affected in branch H1, but 5 (25%) of 20 females were also affected.

The most obvious explanation for the low-penetrance branches in the TAS1 pedigree is heteroplasmy of the 11778 mutation in the early generations. The pathogenic mutation could have segregated into both homoplasmic mutant and homoplasmic wild-type branches (this situation has occurred in the QLD2 11778 LHON family, as described in Howell et al. 1995, p. 298). To test this possibility, we analyzed DNA from seven members of

low-penetrance branches and from two members of high-penetrance branches. In brief, our approach involves both PCR amplification of short (300–400 bp) spans of the mitochondrial genome and subsequent sequencing analysis of multiple, independent M13 clones that contain the mtDNA insert (e.g., see Howell et al. 1991, 1995). For these nine TAS1 LHON family members, the DNA sequences of >400 independent mtDNA inserts that contained a short segment of the ND4 gene were determined. It was found that *all* of them carried the 11778 mutant allele. Furthermore, restriction-site assays of another 40 TAS1 LHON family members have confirmed that the 11778 primary mutation is homoplasmic in all family members (data not shown). Furthermore, tissue-distribution studies indicate that mutation load in blood either reflects the levels in other tissues (Juvonen et al. 1997) or is *lower* than those in other tissues (Howell et al. 1994). Thus, the cumulative results show that the low penetrance in some branches of the TAS1 family is not due to segregational loss (or reversion) of the 11778 mutant allele.

We then extended the sequencing analysis to search for a second site, or suppressor, mitochondrial gene mutation. Family members from the low-penetrance branches may carry a secondary mutation that phenotypically suppresses the pathogenic effects of the 11778 mutation. For example, a suppressor mutation might have arisen in a common maternal ancestor, persisted in the heteroplasmic state for several generations, and eventually become fixed in some branches of the matrilineal pedigree, but not in others, as a result of segregation in the germ line. There are results that suggest the occurrence of mitochondrial suppressor mutations. Thus, the QLD1 LHON family carries, at nucleotide 4160 of the ND1 gene, a mutation that is associated with the severe neurological abnormalities (Howell 1994). A putative intragenic suppressor mutation at nucleotide 4136 has arisen in one small branch (Howell et al. 1991). In addition, Hammans et al. (1995) and El Meziane et al. (1998) have reported suppressor mutations of a pathogenic tRNA mutation.

Six overlapping, PCR-amplified fragments of the mitochondrial genome, which cumulatively spanned nucleotides 10435–12373 (numbered according to Anderson et al. 1981), were analyzed for each of the five TAS1 LHON family members. This 1.9-kb span of the mtDNA included the 3′ half of the $tRNA^{Arg}$ gene, the ND4L gene (nucleotides 10470–10763), the ND4 gene (nucleotides 10760–12137), a cluster of three butt-joined tRNA genes ($tRNA^{His}$, $tRNA^{Ser[AGY]}$, and $tRNA^{Leu[CUN]}$), and the first 36 nucleotides of the ND5 gene. Multiple ($\geqslant$10) independent clones were sequenced for each of the six mtDNA fragments and for each family member, in an effort to detect heteroplasmic mutations. No new sequence changes were detected in any of the five family members. The sequence of this span of the mitochondrial genome was identical for all family members, including the presence of a rare, silent polymorphism at nucleotide 11788. Among the >200 pedigrees (control and LHON) that we have screened, this polymorphism thus far is unique to the TAS1 LHON family, and we have thus verified that the members of the low-penetrance branches are indeed of the correct maternal lineage.

Finally, we have begun a wider search for an *intergenic* mitochondrial suppressor mutation. The first fragment that we analyzed, which spanned nucleotides 3286–3564, included the site of the primary LHON mutation, at nucleotide 3460; the second fragment that we analyzed, which spanned nucleotides 4027–4294, included the sites of both the pathogenic mutation, at nucleotide 4160, and the putative suppresser mutation, at nucleotide 4136, as well as that of the putative secondary LHON mutation, at nucleotide 4216 (Johns and Berman 1991); the third fragment that we analyzed, which spanned nucleotides 14381–14699, included the site of the primary LHON mutation, at nucleotide 14484, and several other sites at which pathogenic mutations have been identified (see the discussion in Howell et al. 1998). The TAS1 mtDNA does not carry any of the aforementioned "accessory" LHON mutations, and there were *no* new mutations in these regions of the mitochondrial genome, among any of the low- and high-penetrance family members who were analyzed.

In addition to the results for the TAS1 LHON family, there are other examples of low-penetrance branches in LHON families. We have also observed that low-penetrance branches apparently occur in the large 14484-mutation TAS2 LHON family (D. A. Mackey, unpublished data), which comprises ~700 maternally related individuals (Mackey and Buttery 1992). As one example, none of the 28 males ($\geqslant$30 years of age) who have descended from female VII-22 have lost vision (authors' unpublished data). We are continuing our analysis of the TAS2 pedigree, because penetrance in 14484-mutation LHON families is more difficult to quantitate with acceptable certainty, because of the high frequency of vision recovery. It becomes more difficult to distinguish a true lack of vision loss from a mild vision loss and rapid recovery, particularly when one must rely, in part, on second-hand information about vision status in relatives. Inspection of pedigree data in the literature also suggests the presence of low-penetrance branches that have been unremarked until now (see, especially, pedigrees XX and XXVIII in van Senus 1963).

Overall, therefore, it appears that low-penetrance branching in LHON matrilineal pedigrees is a biologically "real" phenomenon. One explanation is that the low-penetrance branches are real but that there are dif-

ferent epigenetic and/or environmental factors that lower the penetrance in each branch. Alternatively, low-penetrance branching may be due to the introduction of a *nuclear* genetic suppressor locus. This explanation, however, is problematic, because each low-penetrance branch involves a number of outbreeding events (i.e., marriages), which should act to "localize" any effects of a dominantly acting nuclear locus to one or two generations. Third, low-penetrance branching may be caused by a *mitochondrial* suppressor locus, but one that lies in a mitochondrial genome region that was not sequenced in the experiments that are reported here. Thus far, we have sequenced (*a*) only approximately one-third of the mitochondrial genome that encodes the seven subunits of complex I (NADH-ubiquinone oxidoreductase) or (*b*) only approximately one-fifth of the entire coding region.

The suggestion of a mitochondrial mutation that decreases penetrance in the TAS1 LHON family converges with the related issue of phylogenetic clustering. Both the 11778 mutation and, especially, the 14484 LHON mutation occur more often in European haplogroup J mtDNA backgrounds than would be expected on a random basis (although the TAS1 mtDNA haplotype does not belong to this haplogroup). There is debate over the basis of this clustering phenomenon (see the discussion in Howell et al. 1995 and Mackey et al. 1998), but Brown et al. (1997) and Torroni et al. (1997) have concluded that LHON penetrance is influenced by the mtDNA background in which the pathogenic mutations arise. Thus, the apparent underrepresentation of some mtDNA haplotypes among LHON patients is caused by low penetrance, because of one or more sequence changes within these mtDNAs. As a consequence of the lower penetrance, fewer pedigrees come to the attention of clinicians. Within the haplotype J mtDNA, the site(s) that influences penetrance has not been identified, but the basic premise is similar to that proposed here to explain the presence of low-penetrance branches within a single LHON pedigree. In summary, the present results underscore both the complex etiology of LHON and the fact that the identification of the secondary etiologic factors is a prerequisite for a further understanding of this disorder.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for LHON [MIM 535000])

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Double Heterozygotes for the Ashkenazi Founder Mutations in BRCA1 and BRCA2 Genes

To the Editor:

Three Jewish founder mutations, 185delAG and 5382insC in BRCA1 and 6174delT in BRCA2 genes, have been identified in breast cancer (BC) and ovarian cancer (OC) Ashkenazi patients. In the Ashkenazi general population, the carrier frequencies of these founder mutations are 1% for 185delAG (Struewing et al. 1996), 0.13% for 5382insC, and 1.35% for 6174delT (Roa et al. 1996; Oddoux et al. 1996). Given these high population frequencies, one would expect to find individuals homozygous for the mutations 185delAG/185delAG, 6174delT/6174delT, and 5382insC/5382insC, compound heterozygous for 185delAG/5382insC, or double heterozygous for 185delAG/6174delT or 5382insC/ 6174delT, provided the individuals are viable. The effect of two mutations in a single individual is important both for an understanding of the mode of action and interaction between the BRCA1 and BRCA2 genes and for appropriate genetic counseling. To date, two double heterozygous patients (185delAG/6174delT; Ramus et al. 1997; Gershoni-Baruch et al. 1997) and one patient homozygous for a mutation in exon 11 of the BRCA1 gene (Boyd et al. 1995) have been reported.

By pooling results from four cancer/genetics centers in Israel, we have analyzed ~1,500 BC/OC Ashkenazi patients. All subjects received genetic counseling and signed informed consent forms in compliance with institutional ethics committees (institutional review boards). Each patient was tested for the three Ashkenazi founder mutations: in BRCA1, the mutations 185delAG and 5382insC, and in BRCA2, the mutation 6174delT (Abeliovich et al. 1997; Levy-Lahad et al. 1997; Bruchim Bar-Sade et al. 1998). Four patients were found to be double heterozygotes. Summaries of their clinical status and pedigrees are presented in table 1 and figure 1.

Patient 1 is an Ashkenazi mother of two children who was diagnosed with unilateral breast cancer at the age of 38 years. Her family history was positive for both OC, with which her mother was diagnosed at the age of 50 years, and breast cancer, with which her paternal aunt was diagnosed at the age of 60 years and her daughter at the age of 35 years. Her paternal grandfather had lung cancer at the age of 45 years. A test for 185delAG/ 6174delT in her father revealed neither mutation; DNA could not be retrieved from the paraffin block of her mother. Analysis of the polymorphic markers D17S855, D17S1322, D17S1323, D9S55, and D11S1337 in the father and in Patient 1 confirmed paternity. It was thus